Original Article

Endothelial Progenitor Cell Differentiation and Senescence in an Angiotensin II–Infusion Rat Model

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The ability of endothelial progenitor cells (EPCs) to participate in endothelial repair is impaired by angiotensin II (Ang II) and other atherogenic factors. Therefore, we investigated the effects of Ang II on the differentiation and senescence of EPCs derived from bone marrow (BM-EPCs) in an Ang II–infusion rat model. Wistar rats (*n*=40) were infused with Ang II or vehicle, either alone or in combination with an Ang II type 1 receptor (AT_1R) blocker (valsartan). Bone marrow cells were obtained from the tibias and femurs. Rats of the Ang II treatment group had a significantly lower number of differentiated, adherent BM-EPCs than those of the non-treated control group. Addition of valsartan restored the level of attached, differentiated BM-EPCs to the level in the non-treated controls. The number of senescent BM-EPCs, as assessed by acidic β-galactosidase staining, was significantly greater in the Ang II–alone group than the control group, and addition of valsartan dramatically delayed the senescence of BM-EPCs in the Ang II–alone group. A polymerase chain reaction (PCR)-ELISA–based assay revealed that telomerase activity was significantly lower in BM-EPCs from the Ang II–alone group than in those from the control group, and addition of valsartan significantly augmented this activity. An MTS assay revealed that Ang II treatment significantly decreased the functional activity in BM-EPCs, and this effect was significantly reversed by valsartan. In conclusion, Ang II decreased the differentiation and accelerated the senescence of BM-EPCs *via* AT_1R. (*Hypertens Res* 2006; 29: 449–455)

***Key Words*: endothelial progenitor cells, angiotensin II, senescence, hypertension**

Introduction

The integrity and functional activity of the endothelial monolayer play a crucial role in the prevention of atherosclerosis in hypertension. Recent findings suggest that the injured endothelial monolayer is regenerated by circulating bone marrow (BM)–derived endothelial progenitor cells (BM-EPCs), which accelerates re-endothelialization and limits atherosclerotic lesion formation (*1–4*). This beneficial property of BM-EPCs makes them attractive for cell therapy targeting the regeneration of ischemic tissue. Since BM-EPCs may contribute to postnatal neovascularization, an improved understanding of the regulation of EPC kinetics may provide new insights into the pathogenesis of vasculogenesis. On the other hand, various risk factors for coronary artery disease (CAD), such as diabetes, hyperlipidemia, hypertension, and smoking, affect the number and functional activity of EPCs in healthy volunteers (*5*) and in patients with CAD (*6*). By multivariate analysis of various risk factors, hypertension has been identified as a major independent predictor for impaired EPC migration (*6*). In addition, Min *et al*. have demonstrated that angiotensin-converting enzyme inhibitor therapy with ramipril augmented circulating EPCs with enhanced functional

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Table 1. Characteristics of Each Group

	Control ($n=8$)	Valsartan ($n=8$)	Angiotensin II ($n=8$)	Angiotensin II +hydralazine ($n=8$)	Angiotensin II +valsartan ($n=8$)
BW	371±11	378±22	376±18	360±19	380±17
SBP (mmHg)	110±12	107±7	126±10*	103±13	111±8
HR (/min)	464±39	473±60	478±45	515±22*	461±31

BW, body weight; SBP, systolic blood pressure; HR, heart rate. Data shown are mean±SEM. *Significant difference compared to control, $p<0.1$.

activity in patients with stable CAD (*7*). Given the important role of EPCs for neovascularization of ischemic tissue, a decrease in the number and activity of EPCs could contribute to an insufficient regeneration of the endothelium, which could lead to endothelial dysfunction.

In our recent culture study, we showed that Ang II increases the rate of senescence of EPCs, and that this appears to be a consequence of its ability to stimulate gp91phox expression and thus superoxide formation (*8*). We have also demonstrated that the ability of Ang II to induce senescence involves the suppression of telomerase, which is known to be a key player in the determination of cell life span (*8*). However, it remains unclear whether Ang II affects the differentiation and senescence of BM-EPCs *in vivo*. Therefore, in the present study, we investigated the influence of Ang II on the differentiation and senescence of BM-EPCs in an Ang II–infusion rat model.

Methods

The Ethics Committee for Animal Experiments at the Wakayama Medical University approved the experimental protocol used in this study. The experiment was performed according to the Guidelines for Animal Experimentation at the Wakayama Medical University.

Animals and Protocol

Thirty-two male Wistar rats aged 10 weeks were housed in a room under constant-temperature and a 12-h light/12-h dark cycle. The rats were infused subcutaneously by mini-osmotic pumps (model 2002; Alza Corporation, Mountain View, USA) with Ang II (0.084 mg/kg/day) or vehicle either alone or in combination with an Ang II type 1 receptor (AT_1R) blocker (valsartan; 0.3 mg/kg/day) or hydralazine (10 mg/kg/day). Valsartan was supplied by Novartis Pharmaceutical Company (Basel, Switzerland). These treatments were performed for 2 weeks before preparation. Blood pressure and heart rate were measured at 2 weeks before and after preparation. Systolic blood pressure (SBP) was measured by tail-cuff plethysmography according to the procedure described by Bunag *et al*. (*9*).

Isolation of Mononuclear Cells and Cell Culture

BM cells were obtained from the tibias and femurs of 12-week-old male Wistar rats. BM-derived mononuclear cells (BM-MNCs) were isolated by density gradient centrifugation with Histopaque 1083 (Sigma Chemical Co., St. Louis, USA). After purification with 3 washing steps, 10×10^6 BM-MNCs were plated on vitronectin-coated 6-well plates. Cells were cultured in endothelial basal medium-2 (EBM-2) (Clonetics, Walkersville, USA) with supplements (3 μg/ml bovine brain extract, 30 μg/ml gentamycin, 50 μg/ml amphotericin B, 10 μg/ml human epidermal growth factor, and 5% fetal bovine serum [FBS]). In addition, peripheral blood mononuclear cells (PB-MNCs) were isolated from male Wistar rats treated with Ang II or vehicle either alone or in combination with valsartan at two time points as described above.

Telomeric Repeat Amplification Protocol Assay

For quantitative analyses of telomerase activity, a telomeric repeat amplification protocol (TRAP) assay, in which the telomerase reaction product is amplified by polymerase chain reaction (PCR), was performed using a *TeloTAGGG* PCR ELISA^{PLUS} kit (Roche Molecular Biochemicals, Mannheim, Germany) according to the manufacturer's protocol as previously described (*8*, *10*).

Proliferative Activity Assay

Mitogenic activity was assayed using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenol)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (CellTiter 96 AQ; Promega, Madison, USA). BM-endothelial cell (EC)–like cells were harvested 7 days after culture and re-seeded with a 96-well plate (1×10^4 cells) in 0.1 ml of EBM-2 medium supplemented with 0.5% bovine serum albumin (BSA) in the presence of human recombinant vascular endothelial growth factor (VEGF) (100 ng/ml; R&D Systems, Minneapolis, USA) overnight. After 24 h in culture, MTS/phenazine methosulfate (PMS) solution was added to each well for 3 h, at which time the light absorbance at 490 nm was detected using an ELISA plate reader (Bionetics Laboratory, Kensington, USA).

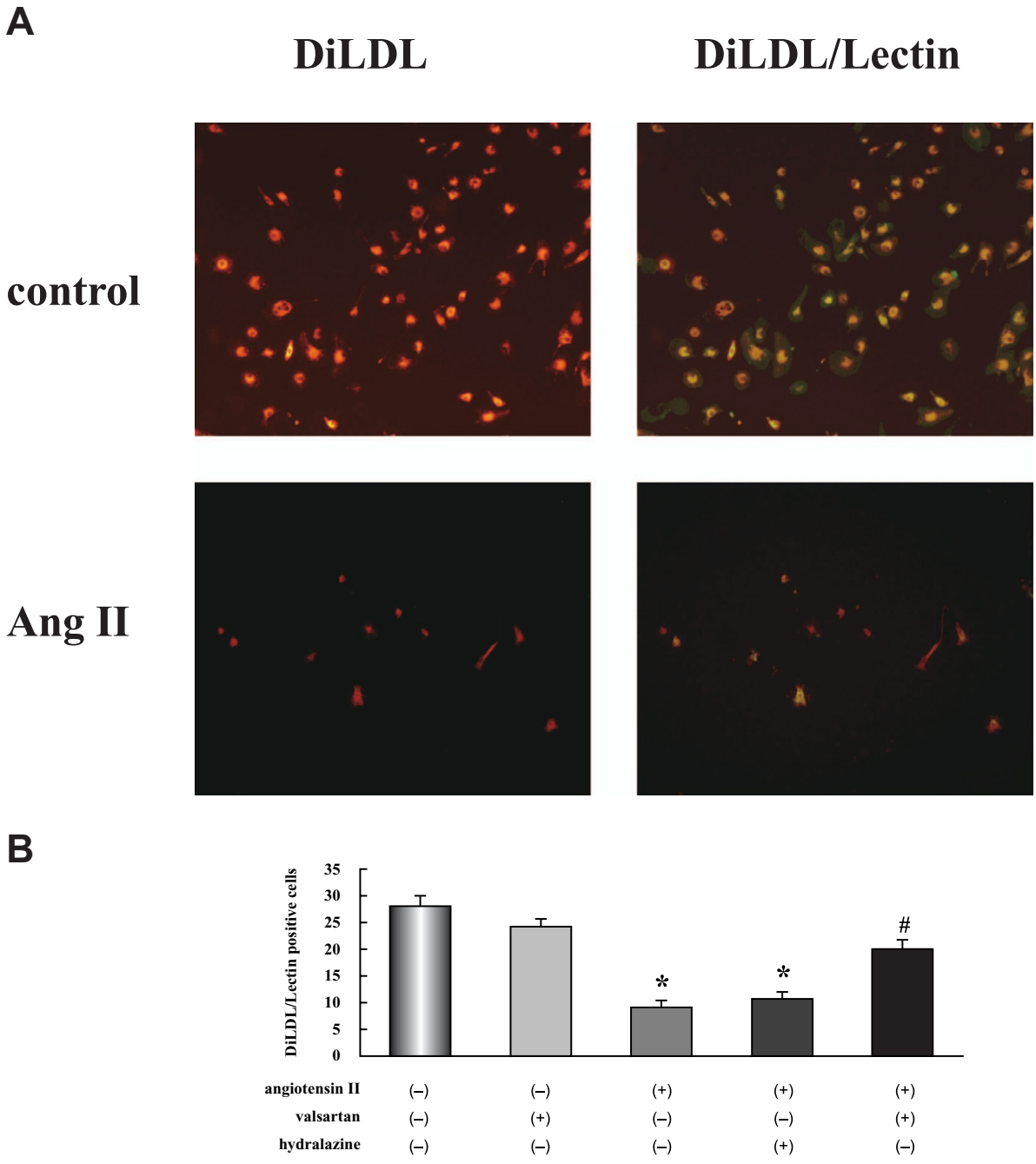

Fig. 1. *Effects of angiotensin II (Ang II) on the differentiation of BM-EPCs. A: The differentiation of BM-EPCs was assessed by double-positive cells for DiLDL uptake and lectin binding. These double-positive cells appear in yellow in the overlay. Representative images treated with (lower) or without (upper) Ang II are shown. B: Quantification of BM-EPC differentiation was evaluated in five groups: the control, Ang II–alone, valsartan-alone, Ang II + valsartan, and Ang II + hydralazine groups. Data are expressed as the means ± SEM (*$n = 8$ *in each group).* *$p < 0.01$ *compared with the control.* #$p < 0.01$ *compared with the Ang II–alone group.*

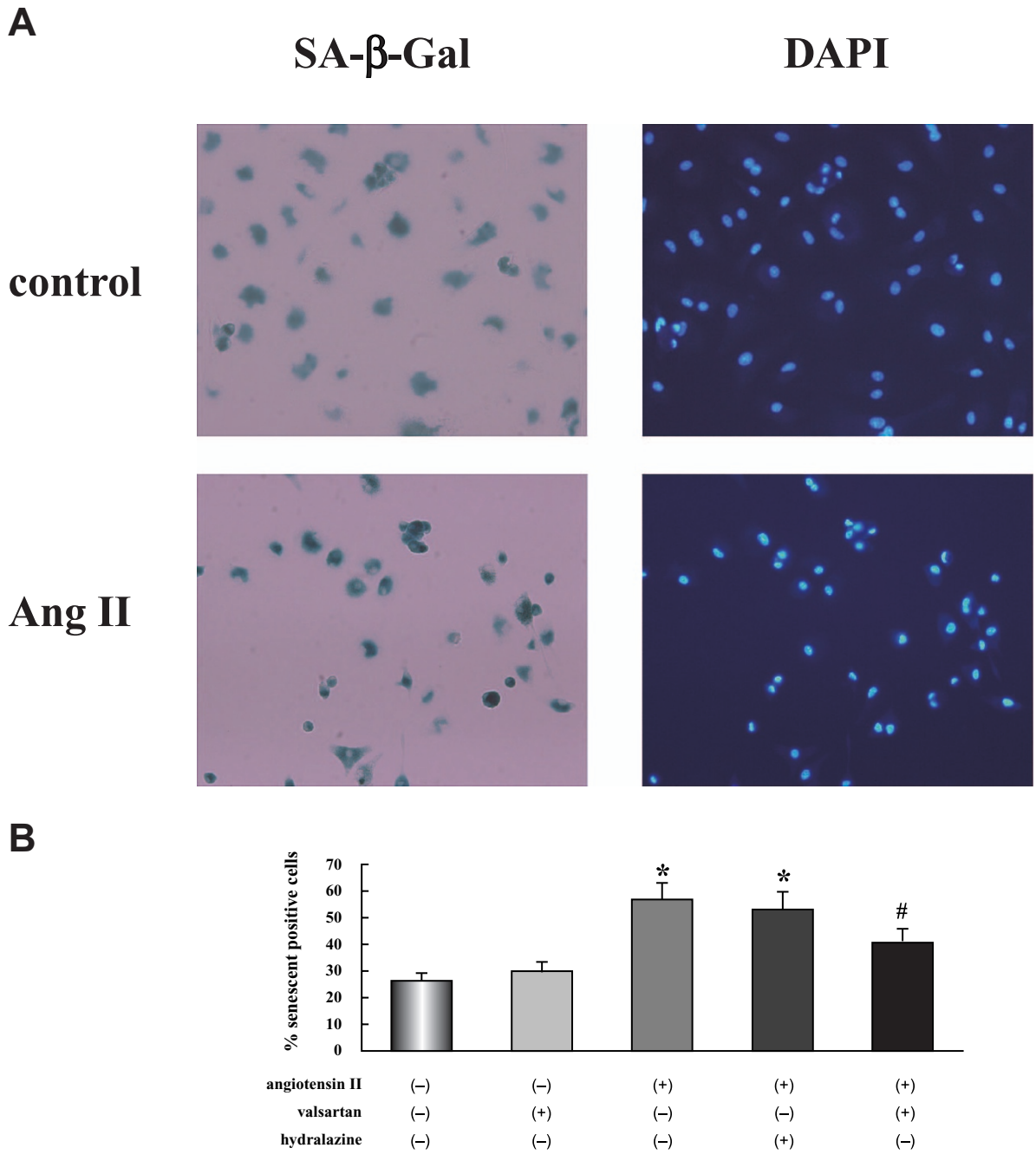

Fig. 2. *Effects of angiotensin II (Ang II) on the senescence of BM-EPCs. A: The senescence of BM-EPCs was assessed by senescence-associated β-galactosidase (SA-β-gal) activity. Representative photomicrographs show SA-β-gal–positive cells (blue) in BM-EPCs treated with (lower) or without (upper) Ang II at day 14. B: Quantification of SA-β-gal–positive cells was evaluated in five groups: the control, Ang II–alone, valsartan-alone, Ang II + valsartan, and Ang II + hydralazine groups. Data are expressed as the means ± SEM (*n = 6). *$p < 0.01$ compared with the control. #$p < 0.01$ compared with the Ang II–alone group.*

Statistical Analysis

Data were expressed as the mean ± SEM based on at least five independent experiments. Statistical analysis was performed by Student's *t*-test. Values of $p < 0.05$ were considered to indicate statistical significance.

Results

SBP was significantly higher in the Ang II treatment group

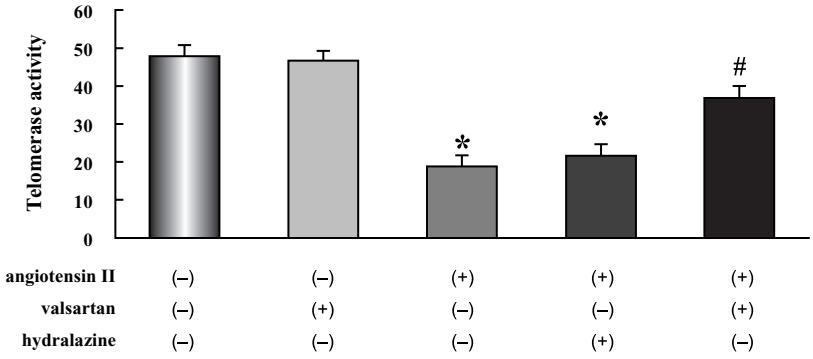

Fig. 3. *Effects of angiotensin II (Ang II) on telomerase activity in BM-EPCs. Freshly isolated mononuclear cells were cultivated in endothelial basal medium-2 (EBM-2) supplemented with EBM-2 simple aliquots. The six-well plates were replated at day 4. After an additional 3 days of cultivation, telomerase activity was measured by a PCR-ELISA–based assay. Data are expressed as the means ± SEM (*$n = 8$ *in each group).* *$p < 0.01$ *compared with the control.* #$p < 0.01$ *compared with the Ang II–alone group.*

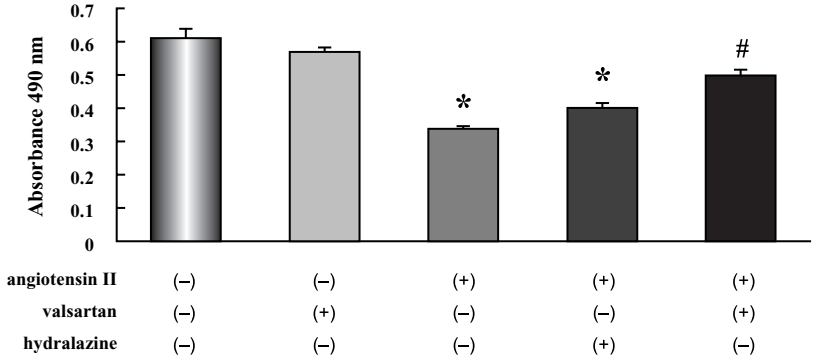

Fig. 4. *Effects of angiotensin II (Ang II) on the proliferative activity of BM-EC–like cells. BM-EC–like cells treated with or without Ang II were harvested 14 days after culture. The mitogenic activity was detected as described in the Methods. Data are expressed as the mean ± SEM (*$n = 6$*).* *$p < 0.01$ *compared with the control.* #$p < 0.01$ *compared with the Ang II–alone group.*

than in the Ang II + valsartan treatment group, the Ang II + hydralazine treatment group, or the control group (Table 1).

BM-EPCs were characterized as adherent cells that were double-positive for both lectin and DiLDL uptake. The number of differentiated, adherent BM-EPCs in the Ang II treatment group was significantly lower than that in controls (Fig. 1). Addition of valsartan significantly reversed this effect (Fig. 1). On the other hand, hydralazine had no effect on BM-EPCs.

The rate of senescence, as assessed by acidic β-galactosidase staining, was significantly higher in BM-EPCs in the Ang II treatment group than in controls or the Ang II + hydralazine treatment group (Fig. 2). On the other hand, Ang II–induced senescence of BM-EPCs was significantly inhibited by concomitant administration of valsartan (Fig. 2). Cellular senescence is critically influenced by telomerase, which elongates telomeres, thereby counteracting the telomere length reduction induced by cell division. Therefore, we measured telomerase activity by a *TeloTAGGG* telomerase PCR ELISA. As demonstrated in Fig. 3, telomerase activity in BM-EPCs was significantly lower in rats treated with Ang II alone than in the Ang II + valsartan treatment or control groups.

Having demonstrated that Ang II accelerated the onset of senescence, we next examined whether this effect translates into a decrease in proliferation. An MTS assay demonstrated that the mitogenic activity in BM-EPCs of the Ang II treatment group was significantly lower than that in the untreated control group, and this effect was significantly reversed by valsartan (Fig. 4).

Finally, we investigated the effect of Ang II on the number of PB-EPCs. As shown in Fig. 5, Ang II significantly decreased the number of PB-EPCs. In fact, the number of PB-EPCs at 1 and 2 weeks after the Ang II treatment was significantly decreased compared with that in the control group, and this effect was significantly inhibited by the co-treatment with valsartan.

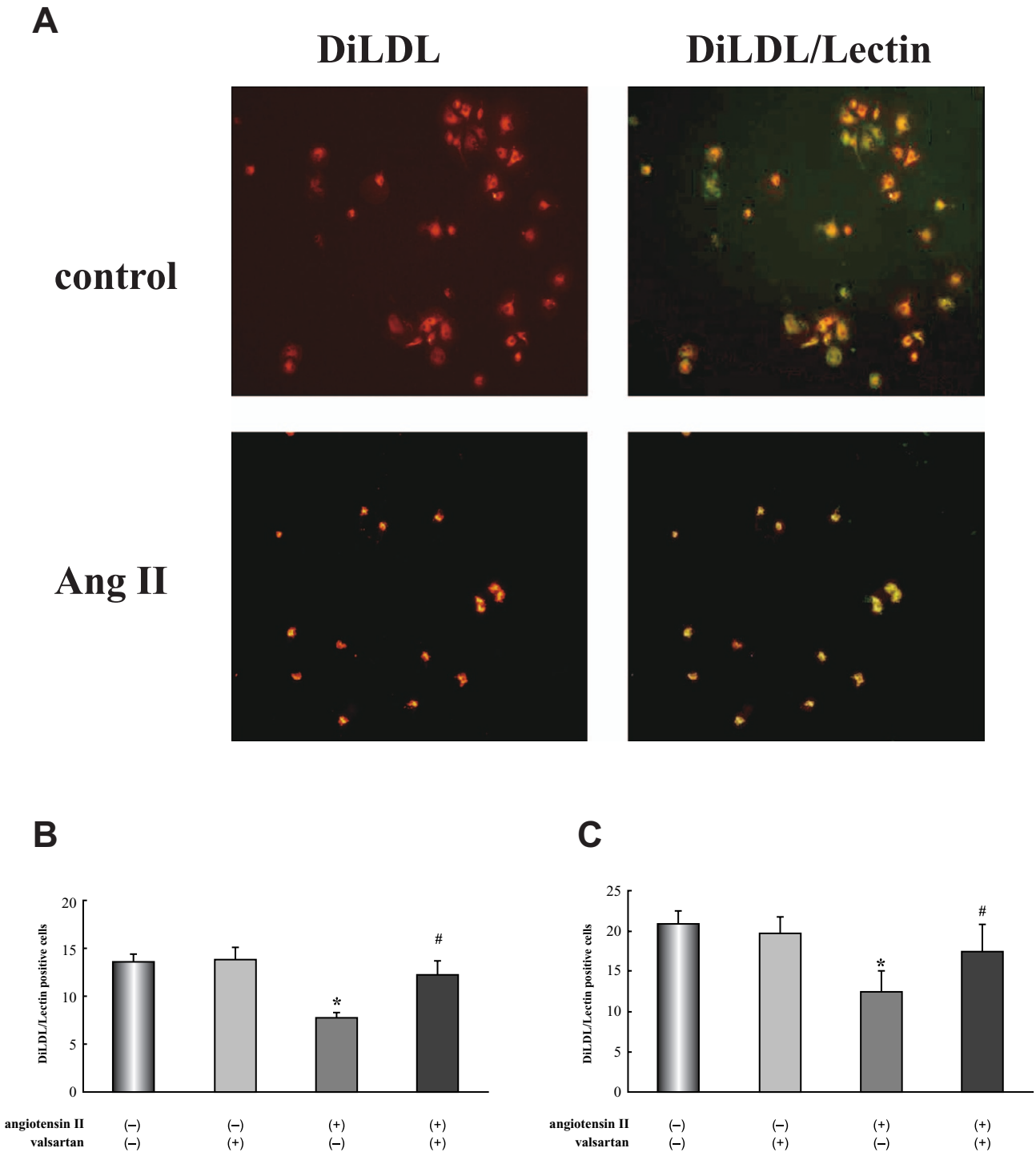

Fig. 5. *Effect of angiotensin II (Ang II) on the number of PB-EPCs. A: The effect of Ang II on PB-EPCs was assessed by double-positive cells for DiLDL uptake and lectin binding. Double-positive cells appear in yellow in the overlay. Representative images of PB-EPCs treated with (lower) or without (upper) Ang II are shown. Quantification of PB-EPCs at 1 week (B) or 2 weeks (C) after Ang II treatment was evaluated in four groups: the control, Ang II–alone, valsartan-alone, and Ang II + valsartan groups. Data are expressed as the means ± SEM (*n = 5 in each group). *$p < 0.05$ compared with the control. #$p < 0.05$ compared with the Ang II–alone group.*

Discussion

The functional regeneration of ischemic tissue by improved neovascularization and subsequent tissue repair is critically dependent on the mobilization and integration of EPCs into the ischemic tissue. Moreover, infusions of EPCs expanded *ex vivo* can limit scar extension in the ischemic myocardium (*11*) and improve the recovery of contractility, and thus may be useful as a novel therapeutic approach (*12*). However, it is conceivable that unfavorable clinical situations might be associated with dysfunctional BM-EPCs, defective vasculogenesis, and thus, impaired neovascularization. Indeed, Tepper *et al*. (*13*) reported that the proliferation and tube formation of EPCs were impaired in patients with type 2 diabetes compared with normal subjects. In the present study, we have shown that the differentiation of BM-EPCs derived from Ang II–infused rats at 12 weeks of age was significantly lower than that of BM-EPCs from untreated rats. Secondly, the Ang II–infused rats showed a significantly greater number of senescent BM-EPCs than the untreated rats. These effects were thought to be mediated *via* AT_1R. Thirdly, Ang II significantly decreased the functional activity of BM-EPCs. Finally, the Ang II group had a significantly smaller number of PB-EPCs than the untreated rats. These results suggest that an AT_1R blocker might have potential for the treatment of postnatal vasculogenesis of ischemic tissue.

Repair of the vessel wall and generation of new blood vessels requires BM-derived stem cells or EPCs. In diseases of the vessel wall, such as atherosclerosis, the number of EPCs is reduced by 40% and their function is impaired (*6*). By multivariate analysis of various risk factors, hypertension has been identified as a major independent predictor for impaired EPC migration. Unlike pluripotent stem cells, which are self renewing, EPCs have a defined life-span and, when they reach the Hayflick limit of telomere shortening that accompanies each mitotic event, they undergo replicative senescence. We have recently demonstrated in a culture study that short-term exposure to Ang II potentiates VEGF-induced proliferation through the up-regulation of VEGF receptor kinase domain–containing receptor (KDR) (*14*), while long-term exposure to Ang II inhibits this proliferation by inactivating telomerase and thereby accelerating the onset of EPC senescence. In the present study, infusion of Ang II for 14 days, like long-term exposure of Ang II *in vitro*, led to the acceleration of BM-EPC proliferation. Since antioxidant and statin treatment, by impeding reactive oxygen species (ROS) production, can delay senescence, and angiotensin-converting enzyme (ACE) inhibitor (ramipril) treatment increases the number and function of EPCs (*7*), the stimulation of senescence by Ang II *in vivo* as well as *in vitro* might involve its ability to generate ROS as well as its telomerase suppression. However, further studies will be needed to confirm our findings.

In conclusion, the results of the present study demonstrated that Ang II accelerated the onset of senescence in an Ang II–infusion rat model, and that this effect may have been related to telomerase inactivation. Importantly, the inhibition of BM-EPC senescence by an AT_1R blocker may improve the functional activity of BM-EPCs for potential cell therapy.

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